

### **Remarks**

Claims 1-75 are pending. Claims 64-67 have been canceled herein. Claims 1-9, 68, and 69 have been canceled and the subject matter presented as new claims 76-89. Claims 10-63 and 70-75, drawn to non-elected inventions, have been withdrawn from consideration. Claims 1-9 and 76-89 are under examination.

Support for new claims 76-89 is found in the claims as filed and at page 24, lines 11-31, page 37, lines 29-31, page 38, lines 6-7, the examples, in Tables I-III, FIGS. 1-5, and in claims 1-9, 68 and 69. No new matter has been introduced by way of these amendments.

### Response to Claim Objections

The Examiner alleges at page 5 of the Office Action that claims 4, 5, 6, 66 and 67 are of improper dependent form because they recite peptides comprising one of the group of D-isomer, or L-isomer, or a combination of D and L isomer amino acids. The Examiner alleges that these claims do not further limit claims 1-3, 64 or 65. The Examiner further alleges that because glycine lacks a chiral center a peptide comprising all glycine residues would not meet the limitations of claims 1-3, 64 and 65. Claims 64-67 have been canceled, therefore the rejection as to these claims is now moot. Claims 1-3 have been cancelled and the subject matter of those claims incorporated into new claims 76 and 82.

A peptide of the invention as claimed cannot consist of "only" glycine residues. The "B" position in the peptide formulas as claimed can only be arginine, lysine, or a combination of arginine and lysine.

The subject matter of former dependent claims 4, 5, and 6, is now incorporated into new dependent claims 80, 81, 86, and 87, which do not recite the phrases "a combination of D- and L-isomer amino acids" and "L-isomer amino acids." Therefore, new dependent claims 80 and 81, and 86 and 87 further limit new independent claims 76 and 82, respectively.

Applicants submit that this amendment has overcome the objections as to new claims 81, 82, 86, and 87.

Response to 35 U.S.C. § 112, second paragraph rejection

Claims 1-9 and 64-69 stand rejected as allegedly indefinite. Claims 64-67 have been cancelled, therefore, the rejection as to these claims is now moot. Claims 1-9 have been canceled herein and their subject matter incorporated into new claims 76-87. Claims 68 and 69 have been canceled and the subject matter incorporated into new claims 88 and 89, respectively.

The Examiner asserts that claims 68 and 69 recite a "nonconcatameric peptide," but because there are repeating units in the peptides, it is unclear how the limitation of "nonconcatameric" can be applied to the peptides.

For clarification purposes, claims 88 and 89 now recite "concatameric" instead of "nonconcatameric." As indicated by the Examiner, repeating units are recited, thus the peptides are "concatameric" by nature (see also Table I).

The Examiner alleges at page 6 of the Office Action that claims 68 and 69 (replaced by claims 88 and 89) recite "Cardin Sites," but that "Cardin Sites" are not defined in the specification. The Examiner asserts that the Cardin sites appear to be the sequence motifs of XBBXXBX and XBBBXXBX of Cardin and Weintraub (Arteriosclerosis, 1989). The Examiner further asserts that the claims describe synthetic peptides wherein Cardin sites are separated by at least one amino acid and wherein the sequence of the synthetic peptide is at least two of the group of (XBBBXXBX)<sub>n</sub>, (XBXXBBBX)<sub>n</sub>, (XBBXXBX)<sub>n</sub> or (XBXBBX)<sub>n</sub>. The Examiner alleges that the metes and bounds of the claims cannot be determined because it is unclear how "Cardin sites" relate to the recited sequence motifs and if the reversed sequences of XBXBBX and XBXXBBBX would be within the scope of a Cardin site. The Examiner alleges that the specification does not define Cardin Site and that Cardin et al. (Arteriosclerosis, 1989, 9:21-13) does not identify the reversed sequences.

The phrase "wherein Cardin sites are separated by at least one of any amino acid." has been deleted from new claims 88 and 89. Claims 88 and 89 recite: "A synthetic concatameric peptide wherein the sequence of amino acid residues of said peptide is represented by at least two segments selected from the group consisting of XBBBXXBX, XBXXBBBX, XBBXXBX, and XBXBBX, wherein: said peptide does not comprise only XBBBXXBX segments; said peptide does not comprise only XBXXBBBX segments; said peptide does not comprise only XBBXXBX segments; said peptide does not comprise only

XBxBBX segments; each segment is separated from an adjacent segment by at least one of any amino acid residue;". Therefore, the claims as amended now refer directly to the claimed segment motifs and do not use the term "Cardin site" when referring to the segment motif. Applicants assert that the claims are now definite and that new claims 88 and 89 are free of the rejection applied to claims 68 and 89.

The Examiner asserts that the element "a single cysteine residue is within three residues of either an N- or C-terminus, either within a Cardin sequence or extended beyond the Cardin sequence" of claim 69 is indefinite. The Examiner alleges that the metes and bounds of a "Cardin site" cannot be determined for the reasons described above and because it is unclear how "Cardin sequence" relates to "Cardin site."

Claim 69 has been rewritten as claim 89 by deleting the phrase, "either within a Cardin sequence or extended beyond the Cardin sequence" and revising the claim to recite that the cysteine residue is within three residues of either an N- or C-terminus. The location of the cysteine residue is now recited specifically based on its position relative to a peptide terminus, without referring to the motifs as Cardin sequences or sites (see page 24, lines 28-31 and Table I). Therefore, the allegation that the metes and bounds of a Cardin site relative to a Cardin sequence cannot be determined is now moot.

The Examiner further alleges that in claim 69 the relationship of the recited cysteine residue to the Cardin site is unclear because X residues must be alanine or glycine and B residues must be arginine or lysine within the sequence motifs. As stated above, the language regarding Cardin sites does not appear in claim 89, which replaces cancelled claim 69. Furthermore, claim 89 recites "cysteine" as an amino acid which can be in an "X" position of the peptide. This amendment is supported throughout the specification and claims as filed. For example, it is stated at page 5, lines 20-21, that any of the peptides of the invention may contain "any amino acid in the X position." In addition, claims 76 and 82 recite "X is any amino acid," as did canceled claims 7-9. Furthermore, it is stated in the application that a peptide of the invention "may include the presence of a single cysteine residue preferably occupying, but not limited to, a position within a three residue distance of either the C- or N-peptide terminus. . ." (page 5, lines 16-18). See also page 5, lines 20-21, page 7, lines 2-3, page 24, lines 17-18 (SEQ ID NOS:35-38), and page 38, lines 9-10. Therefore, the rejection as to "cysteine" is now moot.

The Examiner alleges at page 6 of the Office Action that the term "synthetic," as recited in claims 1-9 and 64-69, now rewritten as claims 79-89, is unclear with regard to the claimed peptides. The Examiner asserts that it is unclear whether the term includes recombinantly produced peptides, or peptides produced by means of chemical or enzymatic degradation in vitro, or whether the term excludes only those peptides found in nature, or all peptides with the exception of those chemically synthesized from amino acids.

One of ordinary skill in the art would understand that "synthetic," as used in the context of the peptides of the invention, refers to a non-natural peptide, e.g., the synthesis was directed by man. For example, "synthetic" can be defined as "man-made; synthesized in vitro; prepared artificially as opposed to being isolated from natural sources" (Dictionary of Biochemistry and Molecular Biology, 1989, 2<sup>nd</sup> ed., J. Stenesh, John Wiley and Sons, New York; copy provided herewith). Non-natural peptide synthesis techniques disclosed in the application include using recombinant DNA techniques and expression vectors (page 25, line 24). Solid phase peptide synthesis using Fmoc chemistry was also disclosed as a method to prepare peptides of the invention (page 25, line 32 to page 26, line 2; also see examples and Tables I-III). One of ordinary skill in the art would know that, in addition to the synthetic techniques described in the application, a variety of techniques exist for synthesizing peptides with desired sequences.

Applicants assert that the claims are now definite and that claims 76-89 are now free of the rejection applied to claims 1-9 and 64-69.

Response to 35 U.S.C. § 102 rejection

Claims 64 and 66 and 65 and 67 stand rejected as allegedly anticipated by Stevens et al. (WO 93/13119), as evidenced by Accession Nos. AAR42842 and AAR05247, respectively. Claims 64-67 have been cancelled herein, therefore the rejection as to these claims is now moot.

Response to 35 U.S.C. § 103(a) obviousness rejections

Response to rejection of claim 68 (now claim 88)

Claim 68, stands rejected under 35 U.S.C. § 103(a) as being unpatentable over De Boer et al. (J. Biol. Chem., 1992, 267:2264-2268), in view of Cardin et al. (Arteriosclerosis, 1989, 9:21-13). Claim 68 has been canceled herein and its subject matter has been incorporated into new claim 88.

Examiner alleges that it would have been *prima facie* obvious for one of ordinary skill in the art at the time the invention was made to substitute “A” for “G” in any “X” position in the Lys348-Arg361 peptide of De Boer et al., in view of certain teachings of Cardin et al. Applicants respectfully submit that the combination of De Boer and Cardin does not render claim 68 *prima facie* obvious under 35 U.S.C. § 103(a), for the following reasons.

Preliminarily, the three-prong test which must be met for a reference or a combination of references to establish a *prima facie* case of obviousness has not been satisfied in the instant matter. The MPEP states, in relevant part:

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all of the claim limitations. MPEP § 2142.

To support a case of *prima facie* obviousness, a combination of references must: (1) suggest to those of ordinary skill in the art that they should make the claimed invention, and (2) reveal to those of ordinary skill in the art that they would have a reasonable expectation of success. In re Vaeck, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). Both the suggestion and the reasonable expectation of success must be found in the prior art and not in Applicant's disclosure. In re Dow Chemical Company, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). None of these criteria have been met here.

Applicants point out that contrary to the Examiner's assertion that claim 68 recites two sequence motifs (XBBBXXBX and XBBXBX), claim 68 (and new claim 88) in fact

recites four consensus sequence motifs or segments, i.e., two 6-mers (XBBXBX and XBxBBX), and two 8-mers (XBBBXXBX and XBXXBBBX).

The asserted combination of references would not have suggested to one of ordinary skill in the art that they should make the claimed invention. It would not have been *prima facie* obvious to substitute "A" or "G" for any of the positions designated as X in the Lys348 to Arg361 peptide as taught by De Boer. As more fully discussed below, neither De Boer nor Cardin addresses the role or importance of any X position residues.

Cardin does not teach or suggest the use of alanine or glycine and only discusses amino acid residues in general terms with respect to their position on the helical face of the peptide and their effect on charge of the peptide (for example, see page 26, left column, lines 1-20). Although Cardin summarizes the amino acid residues present in various heparin-binding peptides in Tables 3 and 4, Cardin never addresses the role of amino acids in X positions. The Examiner asserts that the inclusion of various X residues in a heparin-binding sequence motif can be "deduced" by their absence from the legend of Table 4 of Cardin. The failure of Cardin to calculate the percentage of amino acids in X positions which are not acidic, basic, or aromatic residues, would not suggest to one of skill in the art that these other amino acids should be used in a peptide. For example, alanine and glycine, neither of which are acidic, basic or aromatic residues, are not the predominant X residues in any of the peptides compiled in Tables 3 and 4 of Cardin.

The ability of a peptide of the invention to bind with heparin is correlated with the ability of the peptide to conform to an  $\alpha$ -helix once it binds with heparin (Figs. 3 and 4). The inclusion of alanine in the X position as an  $\alpha$ -helix stabilizer was based on the result described in the application that once the peptide bound to heparin,  $\alpha$ -helical conformation of the peptide occurred (Figs. 2-4, page 24, lines 3-6, page 38, lines 11-13). The peptides of the present invention only adopt an  $\alpha$ -helical conformation upon interacting with heparin and have virtually no intrinsic  $\alpha$ -helix structure until binding occurs (see page 32, line 4 to page 33, line 7; Figs. 3 and 4).

The concept of alanine acting as an  $\alpha$ -helix stabilizer was not recognized by De Boer or Cardin. De Boer teaches a structurally different peptide than the peptides of the invention. For example, residues 347-353 of vitronectin, part of peptide 2 of De Boer, are in

an  $\alpha$ -helical conformation, even when not bound to heparin (Hileman et al., BioEssays, 1998, 20:156-167, provided in the Information Disclosure Statement as reference AG). Thus, De Boer teaches away from the present invention because its teachings would suggest that a peptide of the invention would not bind to heparin unless it assumed an  $\alpha$ -helical conformation in the absence of ligand.

Because neither De Boer or Cardin discusses alanine or glycine, and because neither reference teaches a role for X position amino acids in peptide binding to heparin or other glycosaminoglycans, it would not have been obvious to "deduce" various X position residues based on Table 4 of Cardin, nor would it have been obvious to substitute "A" or "G" for any of the positions designated as X in the Lys348 to Arg361 peptide of De Boer.

The Examiner alleges that De Boer teaches the peptide of the invention, except where X is alanine or glycine. The Examiner's assertion that peptide 2 of De Boer Fig. 5 contains both the 6-mer and 8-mer Cardin consensus sequence sites (as described in Cardin) is incorrect. One alleged Cardin site of De Boer peptide 2 comprises a Cardin-like 6-mer motif XBBXBX. The second alleged Cardin site of De Boer peptide 2 is not the 6-mer or 8-mer motif of Cardin. Rather, the second alleged Cardin site is a 7-mer which De Boer describes as an XBBBXXB motif. Applicant would like to point out that the 7-mer of De Boer is actually the motif BXBBXXB, not XBBBXXB as described by De Boer. Neither 7-mer motif is a Cardin sequence.

Accordingly, although De Boer peptide 2 has two different binding segment motifs, e.g., a 6-mer and a 7-mer, De Boer does not teach a peptide comprising a multimer of at least two different segment motifs, wherein the segment motifs are a Cardin 6-mer or 8-mer, or the reverse sequences of the Cardin 6-mer and 8-mer. De Boer does not even teach or suggest a peptide comprising two or more 6-mer and two or more 7-mer motifs. Furthermore, because the specific pentasaccharide binding region in heparin is only sparsely distributed throughout the heparin chain, it would have been counterintuitive to expect that a concatameric peptide of Cardin sequences or reverse Cardin sequences would be effective in binding heparin. Therefore, De Boer does not teach a peptide of the invention except where X is alanine or glycine.

Examiner alleges that De Boer teaches that inhibition of binding of the vitronectin-thrombin-antithrombin complex to endothelial cells by heparin-binding peptides is correlated directly with the binding of the peptides to heparin. It is respectfully submitted that Examiner's interpretation of De Boer is incorrect. De Boer Fig. 5 merely demonstrates that certain peptides can inhibit binding of vitronectin-thrombin-antithrombin complexes to endothelial cells. Fig. 5 does not demonstrate that the De Boer synthetic peptides or vitronectin fragments bind to heparin or other glycosaminoglycans or proteoglycans. De Boer presents no data regarding peptide binding to heparin. The De Boer statement referred to by the Examiner regarding heparin-binding (page 2267, second column, bridging sentence to page 2268) merely cites Tomassini et al., (Blood, 1986, 68:737-742; copy provided herewith) and suggests that there may be a correlation between peptide binding to heparin and peptide binding to a cell. In fact, De Boer misstates the relationship between his peptide-cell binding data and the data of Tomassini.

Tomassini shows differences in the ability of serum-derived or plasma-derived vitronectin to bind to heparin-agarose in the presence of other proteins. Tomassini does not provide data on the affinity of vitronectin or any other proteins or peptides for non-derivatized heparin. In addition, Tomassini suggests that vitronectin only binds to heparin-agarose when vitronectin is complexed with thrombin and anti-thrombin III (Tomassini, page 740, column 2). One of ordinary skill in the art would not view as equivalent peptide- or protein-complex binding *to a cell*, as taught by De Boer, and binding of a protein-complex *to a solid-supported heparin* such as heparin-agarose, as taught by Tomassini. The in vitro cellular assay of De Boer is a general assay for detecting the ability of a protein-complex to bind with glycosaminoglycans or proteoglycans. As performed by De Boer, vitronectin-thrombin-anti-thrombin III-complexes bind not just to heparin, but to many different glycosaminoglycans and proteoglycans present on the cell surface or which have been deposited as part of the extracellular matrix. One of ordinary skill in the art would understand that the binding affinity of the protein-complex for the various glycosaminoglycans and proteoglycans varies greatly, and that the De Boer data represent an average binding affinity of the protein-complex for all glycosaminoglycans and proteoglycans which are present. The De Boer assay therefore, does not measure the affinity of the protein-complex for heparin. The solid-supported heparin adsorption assay



used by Tomassini is not a general assay for glycosaminoglycans and proteoglycans. In the solid-supported heparin adsorption assay used by Tomassini, the protein-complex is contacted with heparin conjugated to an agarose support matrix, in the absence of glycosaminoglycans and proteoglycans. Thus, the De Boer assay and the Tomassini assay are not equivalent. For the same reasons, one of ordinary skill in the art would not view a peptide- or protein-complex binding *to a cell* as equivalent to a peptide binding *to a non-derivatized heparin*, where binding to non-derivatized heparin is performed in the absence of other glycosaminoglycans and proteoglycans.

Cardin does not remedy the deficiencies of De Boer. Cardin teaches that B position residues can be many different amino acids, including non-basic amino acids (Tables 3 and 4). For example, Cardin disclosed that one of the B positions of the 6-mer (B-2) and two of the B positions of the 8-mer (B-3 and B-1) comprise amino acid residues other than the basic amino acids arginine and lysine. Therefore, Cardin teaches away from the present invention which claims only arginine and lysine residues in the B position, and therefore does not teach each and every element of claim 88.

Even if De Boer and Cardin were combined, the result is not the claimed invention. Neither reference, teaches a synthetic concatameric peptide wherein the peptide comprises at least two different segments selected from the group consisting of XBBBXXBX, XBXXBBBX, XBBXXBX, or XBXBBX, and further wherein the peptide does not comprise only XBBBXXBX segments, or only XBXXBBBX segments, or only XBBXXBX segments, or only XBXBBX segments. Furthermore, because neither reference teaches or suggests that X position amino acids comprise only alanine or glycine residues or that B positions comprise only arginine or lysine residues, combining the references would not result in the claimed invention.

For the reasons described above, the claimed invention would not have been obvious from the combination of De Boer and Cardin. Applicants assert that new claim 88 is free of the rejection applied to claim 68.

Claims 1, 2, 4, 5, 7, and 8 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Cardin et al., in view of Wakefield et al. (Surgical Research, 1994, 56:586-593) and Fromm et al. (Archives of Biochemistry and Biophysics, 1997, 343:92-100) and Margalit et al. (J. Biol. Chem., 1993, 268:19228-19231). Claims 1, 2, 4, 5, 7, and

8 have been canceled herein and their subject matter incorporated into new claims 76, 77, 78, 79, 80, and 81.

Applicants respectfully submit that the combination of Cardin, Wakefield, Fromm, and Margalit does not render claims 1, 2, 4, 5, 7, and 8 *prima facie* obvious under 35 U.S.C. § 103(a) for the following reasons.

First, it would not have been obvious to combine Cardin with Wakefield, Fromm, and Margalit. As described above, Cardin only discloses two sequence motifs and does not disclose peptides which comprise multimers of consensus sequences. Contrary to the assertion of the Examiner, Cardin teaches that B position amino acid residues are not just basic amino acids. Furthermore, Cardin does not disclose peptides which comprise multimers of consensus sequences.

As described above, it would not have been *prima facie* obvious to substitute "A" or "G" for any of the positions designated as X in the heparin binding consensus sequences as taught by Cardin. Cardin only addresses the role of B position residues in the ability of the peptides to bind heparin. As described above, Cardin does not address a functional role of amino acids in X positions. Cardin merely catalogs amino acids found in X positions. The failure of Cardin to compile neutral or hydrophobic residues in the legend of Table 4 does not suggest that such amino acids should be used in the X positions. Therefore, Cardin does not teach or suggest the use of alanine or glycine or contemplate a role of alanine or glycine in the ability of the peptide to bind with a glycosaminoglycan or proteoglycan.

Wakefield does not remedy the deficiencies of Cardin. Wakefield teaches binding of protamine analogs to heparin, but does not teach or suggest heparin-binding peptides comprising 6-mer or 8-mer consensus sequences.

Furthermore, Wakefield teaches acetylating or amidating protamine peptides to increase stability by  $\alpha$ -helix formation and to decrease toxicity of the peptides. The protamine analog peptides of Wakefield showed increasing toxicity with increasing numbers of basic amino acids, particularly 18 or more basic amino acids. Wakefield disclosed that protamine analogs comprising 16 or more basic amino acids required acetylation or amidation to reduce their toxicity (see abstract). The chemical modifications were also found to increase  $\alpha$ -helix stability of the peptides. Because Wakefield teaches chemical

modification of peptides to improve heparin-binding and  $\alpha$ -helix stability, it teaches away from the present invention. In addition, because Wakefield teaches that only shorter peptides are not toxic, it teaches away from the present invention.

Even if Cardin or Wakefield taught a heparin-binding peptide comprising concatamers of 6-mer or 8-mer consensus sequences, it would not be obvious to combine the two references. As described above, it would be counterintuitive to use a concatameric peptide of Cardin sequences to contact and span a substantial portion of a heparin molecule, because the pentasaccharide binding sequence in heparin is only widely dispersed throughout the molecule. Because neither Cardin nor Wakefield teaches concatameric 6-mer or 8-mer heparin binding sequences, and because Wakefield teaches that only shorter peptides are not toxic, it would not be obvious to combine Cardin and Wakefield.

Although the Examiner cited Fromm in combination with Cardin, Wakefield, and Margalit, no discussion of Fromm was provided by the Examiner. However, Applicants assert that Fromm does not remedy the deficiencies of Cardin and Wakefield as described above. Fromm merely discloses the spacing of non-basic amino acids relative to other amino acids in a heparin-binding peptide (Fromm, Fig. 3). Fromm does not refer to specific non-basic amino acids or their role in peptide conformation or binding to heparin. Fromm does not teach or suggest concatamers of Cardin sequence motifs, or concatamers of any other binding motif.

Margalit does not remedy the deficiencies of Cardin, Wakefield, and Fromm. The Examiner cites Margalit for summarizing known properties of heparin. Margalit states that heparin is a negatively charged polymer and that proteins are expected to bind to it. Margalit in fact teaches away from the present invention. Margalit describes the three-dimensional structure of putative heparin-binding regions of proteins and the spacing and location of basic amino acids in those heparin-binding regions. Furthermore, Margalit teaches the importance of heparin-binding peptides having intrinsic  $\alpha$ -helix or  $\beta$ -sheet structure to be able to bind heparin and teaches that consensus sequences are unimportant for binding with heparin (see abstract and page 19230, column 2). Margalit does not teach or suggest the use of concatamers of any type of binding sequence. The teachings of Margalit suggest that the peptides of the present invention would not have heparin-binding

activity, because the peptides of the invention only assume  $\alpha$ -helical conformation upon binding with heparin. The teachings of Margalit further suggest that the peptides of the invention would not have heparin-binding activity because the peptides of the invention comprise heparin-binding consensus sequences, while Margalit suggests that consensus sequences are unimportant in heparin-binding regions. Because Margalit teaches away from the present invention and because neither Margalit, Cardin, Wakefield, nor Fromm teach concatamers of 6-mer or 8-mer consensus sequence heparin-binding regions, it would not be obvious to one of skill in the art to combine Margalit with Cardin, Wakefield and Fromm.

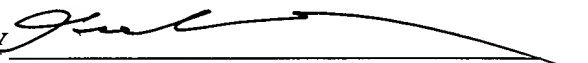
Even if Cardin, Wakefield, Fromm, and Margalit were combined, the resulting combination would not result in the claimed invention. Taken together, these references do not teach or suggest a synthetic peptide, wherein the sequence comprises concatamers of  $(XBBBXXBX)_n$ ,  $(XBXXBBBX)_n$ ,  $(XBBXBX)_n$  or  $(XBXBBX)_n$ . Therefore, the combination of Cardin, Wakefield, Fromm, and Margalit would not result in concatamers as claimed in the present invention. These references do not teach that X is alanine or glycine (claims 1, 2, 4, and 5), that B is arginine, lysine, or a combination of lysine and arginine, or that the peptide can comprise D-isomer amino acids (claims 4 and 5). Therefore, the combination of these references would not result in the claimed invention.

Therefore, based on the reasoning described above, the claimed invention would not have been obvious from the combination of Cardin, Wakefield, Fromm and Margalit. Applicants assert that new claims 76, 77, 78, 79, 80, and 81 are free of the obviousness rejection as applied to claims 1, 2, 4, 5, 7, and 8.

Conclusion

Based on the foregoing, all claims are believed to be in condition for allowance. An early and favorable action toward that end is earnestly solicited.

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## On the Identity of Vitronectin and S-Protein: Immunological Crossreactivity and Functional Studies

By Bianca R. Tomasini and Deane F. Mosher

Vitronectin (serum spreading factor), a major serum cell adhesion molecule, was compared with S-protein, the inhibitor of the C5-9 membrane attack complex. Data from the literature indicate that S-protein and vitronectin are alpha globulins with the same aminoterminal residues, amino acid compositions, and concentrations in normal plasma (150 to 250  $\mu\text{g/mL}$ ). Both proteins have been reported to interact with the thrombin-antithrombin complex. The cDNA sequences of vitronectin and S-protein were recently determined and found to be almost identical. In the present studies, rabbit-anti-S-protein and a monoclonal antibody to vitronectin both recognized 65,000- and 75,000-molecular weight (mol wt) polypeptides when plasma or serum proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The 65,000 and 75,000-mol

wt polypeptides bound more avidly from serum than plasma to monoclonal anti-vitronectin or heparin coupled to agarose. The presence or absence of the polypeptides constituted a major difference between the heparin-binding proteins of serum and plasma. When complement-activated serum and unactivated serum were separated by gel filtration, vitronectin coeluted with C9 in high-mol-wt fractions of activated serum but not unactivated serum. Purified S-protein was recognized by the monoclonal antibody to vitronectin and promoted spreading of human skin fibroblasts. Both vitronectin and S-protein were degraded by thrombin. On the basis of immunological and functional, as well as biochemical, properties, therefore, S-protein and vitronectin are the same.

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**VITRONECTIN (SERUM SPREADING FACTOR)** is responsible for most of the cell spreading activity present in serum.<sup>1</sup> Vitronectin is an alpha globulin<sup>2</sup> that has been isolated in the form of two noncovalently associated polypeptides with molecular weights (mol wt) of 75,000 and 65,000.<sup>3,4</sup> Like the other cell attachment protein of serum fibronectin, vitronectin has been localized in the extracellular matrices of various tissues<sup>5,6</sup> and cells in culture<sup>4</sup> and circulates in plasma at a concentration of 250  $\mu\text{g/mL}$ .<sup>6</sup> The cDNA of vitronectin has been sequenced, and the deduced amino acid sequence contains the tripeptide arg-gly-asn, which is known to be a necessary recognition sequence for cellular adhesion to several adhesive proteins.<sup>7</sup> Recent studies by Pytela et al have described a presumptive cellular adhesion receptor for vitronectin that has two components with mol wt of 125,000 and 115,000.<sup>8</sup>

It was found by Suzuki et al<sup>9</sup> and Barnes et al<sup>10</sup> that the sequence of the first 44 amino acids at the NH<sub>2</sub>-terminal of vitronectin was identical to the sequence of Somatomedin B, a polypeptide previously believed to have growth-promoting capabilities. Due to the relatively high concentration of vitronectin in plasma and serum, it seemed probable to us that vitronectin itself also had been characterized before. Although we compared the properties of vitronectin with a number of 60,000- to 80,000-mol wt glycoproteins, we found no similarities. At the suggestion of Dr John Griffin (Dept of Immunology, Research Institute of Scripps Clinic, La Jolla, Calif), we tested the possibility that S-protein of the complement system might be similar to vitronectin.

S-protein, as characterized by Podack and Müller-Eberhard,<sup>11</sup> is a glycoprotein that binds to C5b-7. The soluble complex then incorporates C8 and C9. By promoting the formation of this soluble complex rather than the lytic membrane attack complex, S-protein is thought to inhibit (modulate) complement-mediated cell lysis. The second role associated with S-protein is that of binding to the thrombin-antithrombin III complex<sup>11-13</sup> in a manner that slows down the formation of the thrombin-antithrombin III complex, thereby resulting in the protection of thrombin from inactivation by antithrombin III. The thrombin-antithrombin III complex, once formed, binds tightly to S-protein.

When we compared various properties of vitronectin and S-protein reported in the literature, we found many similarities. S-protein, like vitronectin, consists of two polypeptides with mol wt in the range of 65,000 and 80,000,<sup>14</sup> is present in plasma at concentrations of 200  $\mu\text{g/mL}$ , and has the electrophoretic mobility of an alpha globulin.<sup>11</sup> It has the same NH<sub>2</sub>-terminal residue as vitronectin.<sup>14</sup> The glass-binding properties of vitronectin have been well documented.<sup>4,15</sup> S-protein has been reported to adsorb extensively onto other surfaces, and thus the yields of published purifications are less than 10%.<sup>14</sup> Ill and Ruoslahti recently found that vitronectin binds to the thrombin-antithrombin III complex in a manner similar to that described for S-protein.<sup>16</sup> Finally, when we compared the reported amino acid compositions of vitronectin and S-protein,<sup>3,14</sup> we found extensive similarities. No residue was different by more than 10%.

In the present paper we show that S-protein and vitronectin are immunologically and functionally the same. While these studies were being prepared for publication, the

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sequence of a cDNA for S-protein was published and shown to be nearly identical to cDNA for vitronectin.<sup>17</sup>

### MATERIALS AND METHODS

**Column chromatography of plasma and serum.** All column chromatography was carried out at room temperature. Platelet-poor fresh frozen plasma was obtained from the Red Cross (Madison, Wis), and serum was obtained after syneresis of clots formed from recalcified citrated plasma (20 mmol/L CaCl<sub>2</sub>).

Chromatography of plasma or serum on monoclonal-anti-vitronectin (MaVN)-Sephacryl column was done as described by Suzuki et al.<sup>9</sup> Hybridoma cells producing MaVN<sup>9</sup> were obtained from Dr E. Hayman (La Jolla [Calif] Cancer Research Foundation), and MaVN IgG was prepared by chromatography of spent media or mouse ascites on protein A-Sepharose (Sigma Chemical Co, St Louis). MaVN IgG was coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. A column of MaVN-Sepharose (2 × 2.5 cm) was equilibrated with phosphate-buffered saline, pH 7.0, before application of plasma or serum (2 mL), and bound material was eluted with 8 mol/L urea, 50 mmol/L Tris, pH 7.0. Protein elution was monitored by absorbance at 280 nm, and peak fractions were collected in polypropylene tubes.

Heparin-agarose (Bio-Rad, Richmond, Calif) columns (1 × 6 cm) were equilibrated with Tris-buffered saline, 1 mmol/L EDTA, pH 7.4. Bound protein from 1 mL plasma or serum was eluted with 600 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4.

As a control, plasma and serum samples were chromatographed on Sepharose 4B columns under conditions described earlier, and effluent volumes corresponding to peak fractions were saved and analyzed.

**Immunoblotting.** Rabbit antihuman S-protein (RaS-protein) and rabbit antihuman C9 (RaC9) were purchased from Calbiochem (San Diego). Samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gels<sup>18</sup> and then electroblotted<sup>19</sup> onto nitrocellulose filters (Schleicher & Schuell, Keene, NH). One section of the blot was stained with 0.1% naphthol blue black in 45% methanol and 10% acetic acid to visualize protein. Replicate sections were soaked in Tris-buffered saline containing 3% bovine serum albumin for one hour at 37 °C, rinsed in Tris-buffered saline, and incubated overnight in Tris-buffered saline containing 1% normal goat serum and 1% appropriate rabbit antiserum or 10% spent medium from MaVN hybridoma cells. After incubation with primary antibody, blots were rinsed in Tris-buffered saline and incubated for one hour at room temperature in Tris-buffered saline containing 1% normal goat serum and 1% peroxidase-conjugated antirabbit or antimouse IgG (Cappel Laboratories, Westchester, Pa). Blots were rinsed again in Tris-buffered saline and washed in substrate solution (Polysciences, Warrington, Pa; Kirkegaard and Perry, Gaithersburg, Md).

**Chromatography of zymosan-activated serum.** Zymosan (Sigma) was suspended in Tris-buffered saline containing 2 mmol/L Ca<sup>++</sup> and 2 mmol/L Mg<sup>++</sup>. Either 0.5 mL of zymosan suspension (100 mg/mL) or buffer alone was added to 5 mL serum, and the mixtures were incubated for four hours at 37 °C in the presence or absence of 20 mmol/L EDTA. Samples were centrifuged at 9,000 g for ten minutes and supernatants (0.5 mL) were separated on Bio-Gel A-15m or A-1.5m columns (1.3 × 18 cm). Columns were equilibrated and run with Tris-buffered saline containing 2 mmol/L Ca<sup>++</sup> and Mg<sup>++</sup> at room temperature. Fractions (0.9 mL) were collected in polypropylene tubes and analyzed by electrophoresis and immunoblotting as described.

**Cell adhesive activity of purified S-protein.** S-protein was purified as described by Dahlbäck and Podack.<sup>14</sup> The purification

was monitored by SDS-PAGE, and at each step, the fractions predicted to contain S-protein contained 65,000- and 75,000-mol wt polypeptides that were recognized by MaVN on immunoblots. Contaminants present in the preparation obtained after the Sephacryl S-200 chromatography step were removed by chromatography on concanavalin A-Sepharose 4B<sup>1</sup> (Sigma) and gelatin-agarose (Sigma). Cell attachment and spreading activity of the final preparation was tested by coating 22-mm-diameter wells in microtiter plates (Costar, Cambridge, Mass) with 0.2 mL of a 10-μg/mL protein solution (one hour incubation). The wells were washed and incubated with 0.3% bovine serum albumin, washed again, and incubated with a 2 × 10<sup>4</sup> cells per milliliter suspension of cultured foreskin fibroblasts (A1-F) in serum-free F-12 media for one hour at 37 °C. Wells coated with fibronectin, vitronectin purified by the method of Hayman et al.,<sup>40</sup> and albumin were used as positive and negative controls. Adherent cells were stained with Coomassie brilliant blue, and replicate 0.6-mm<sup>2</sup> fields were counted.

**Thrombin cleavage of vitronectin and S-protein.** Vitronectin and S-protein (purified as described) were incubated with a 1.6 molar excess of alpha thrombin (a generous gift from John Fenton II, New York State Department of Health, Albany) for two hours at 37 °C, and the reaction was stopped by addition of the synthetic thrombin inhibitor PPACK (Calbiochem). Fragmentation was monitored by SDS-PAGE analysis and immunoblotting with MaVN.

### RESULTS

As shown in Fig 1, RaS-protein was polyspecific when assayed by immunoblotting of plasma and serum; two of the proteins recognized were serum albumin and IgG, reactions we also found with several other commercial antisera. Two major polypeptides were recognized by the antiserum. The two polypeptides migrated with the same mobilities as the polypeptides recognized by MaVN and reacted with the same proportional intensities.

As shown in Fig 2, when plasma (lane 1) and serum (lane 2) samples were chromatographed on a MaVN-Sepharose column (lanes A) on a heparin-agarose column (lanes B), and, as a control, on a Sepharose column (lanes C), the polypeptides recognized by MaVN bound to MaVN and heparin out of serum but not out of plasma. The antiserum to S-protein recognized two polypeptides with identical migration in SDS gels as those recognized by MaVN and with the same proportional intensities. In other studies, we found that if plasma was recalcified in the presence of thrombin inhibitor and then passed over a heparin column, vitronectin did not bind<sup>20</sup> (not shown).

Mimicking experiments reported for S-protein by Podack et al.,<sup>21</sup> we separated zymosan-activated serum or unactivated serum by gel filtration on Biogel A-15m and compared the elution patterns of vitronectin and C9 (Fig 3). The lane numbers in Fig 3B represent every fourth fraction of the elution profile shown in Fig 3A, beginning with the void volume. C9 was identified in high-mol-wt fractions (shown in lanes 2 through 5) of zymosan-activated serum. The equivalent fractions of unactivated serum (lanes 2 through 5) did not contain C9. Monomeric C9 would be expected to elute with albumin; however, the presence of albumin in the fractions shown in lanes 6 through 9 distorts the C9 staining. Immunoblotting of a replicate gel of activated serum with MaVN demonstrated vitronectin in the same high-mol-wt

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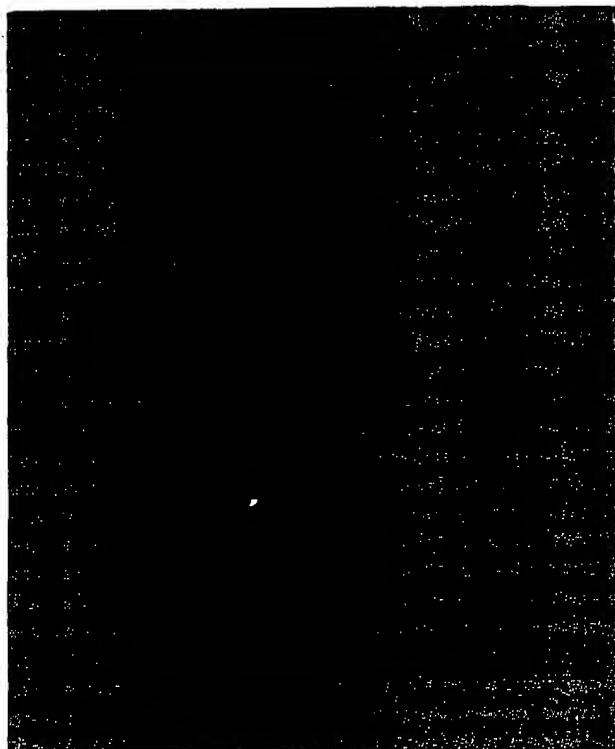


Fig 1. Immunoblots of plasma and serum with MaVN and RaS-protein. Reduced plasma (lane 1) and serum (lane 2) proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters. Part of the blot was stained for protein with 1% naphthol blue black; replica blots were incubated with MaVN (10% spent media) or 1% RaS-protein, followed by the appropriate peroxidase-conjugated secondary antibody to visualize antibodies bound.

fractions in which C9 was detected (lanes 3 through 5). Vitronectin in unactivated serum or in serum treated with EDTA followed by zymosan (not shown) was found only in the fractions containing lower-mol-wt proteins (lanes 5 through 9). That is, vitronectin was present in eight fractions (7.2 mL) of separated activated serum that did not contain vitronectin in unactivated serum (indicated by bar in Fig 3A). The finding that vitronectin co-eluted with C9 in high-mol-wt fractions of zymosan-activated serum was reproducible and also evident when serum samples were chromatographed on Bio-Gel A-1.5m (not shown). RaS-protein, like MaVN, detected the 65,000- and 75,000-mol wt polypeptides in the high-mol-wt fractions of activated but not unactivated serum (not shown).

S-protein purified by the method of Dahlbäck and Podack<sup>14</sup> was tested for spreading factor activity. Trace amounts of fibronectin (approximately 0.2%) and albumin detectable by immunoblotting in the preparation after the Sephacryl S-200 gel filtration step were removed by chromatography on concanavalin A-Sepharose 4B and gelatin agarose. The protein obtained was recognized by MaVN and RaS-protein (not shown) and was active in a cell spreading assay (Table 1).

Podack and Müller-Eberhard<sup>11</sup> reported the lack of susceptibility of S-protein to cleavage with a twofold molar excess of thrombin. In contrast, Silnutzer and Barnes<sup>22</sup> have reported the cleavage of serum spreading factor (vitronectin) to a 57,000-mol wt fragment produced by incubation with thrombin (observed with a 0.15–1.3-fold molar ratio of thrombin to spreading factor) for two hours at 37 °C. As shown in Fig 4, when vitronectin (lane 1) or S-protein (lane 2) was incubated with thrombin (lanes 3 and 4, respectively), a major 60,000-mol wt fragment was obtained (indicated by

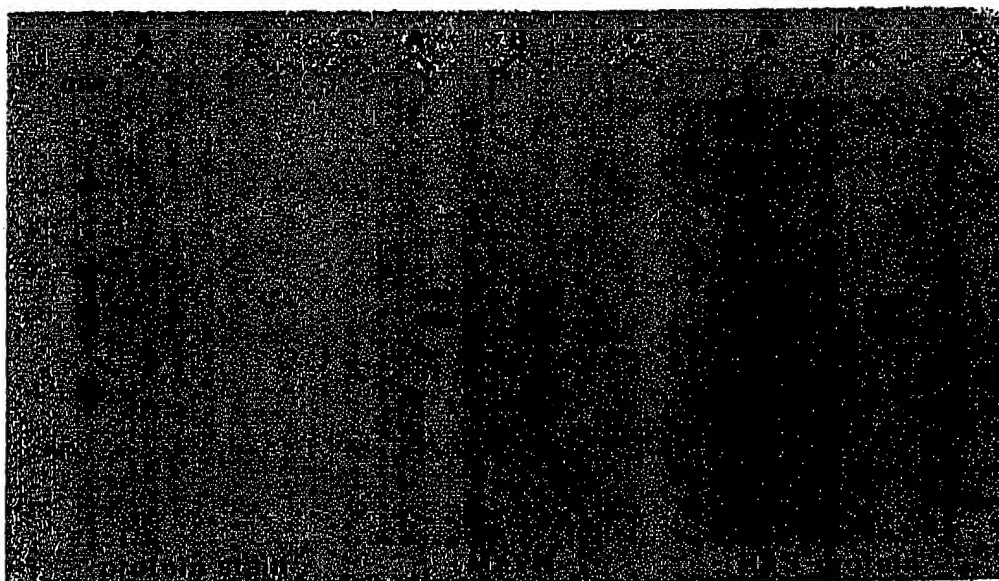


Fig 2. Immunoblots of affinity chromatography-separated plasma and serum against MaVN and RaS-protein. Shown here are the bound fractions of plasma (lanes 1) and serum (lanes 2) chromatographed on MaVN-Sepharose (lanes A), heparin-agarose (lanes B), and Sepharose (lanes C). Samples were analyzed as described in Fig 1.



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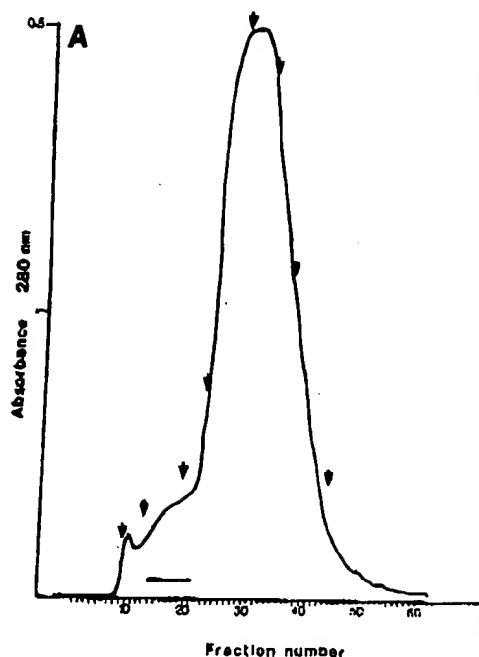


Fig 3. Separation of zymosan-activated serum and unactivated serum by gel filtration on Bio-Gel A-15m. (A) Protein elution profile for 0.5 mL activated serum (the same profile was obtained for unactivated serum). Arrows indicate the positions of fractions used in immunoblotting of both activated and unactivated serum. The line indicates the fractions where VN was detected in activated but not unactivated serum. (B) Immunoblots of fractions from zymosan-activated serum and unactivated serum reacted against MaVN (above) and RaC9 (below), followed by the appropriate peroxidase conjugates.

arrowhead). This fragment was detected by immunoblotting with MaVN and by protein stain (not shown).

#### DISCUSSION

We found that a commercial rabbit antiserum to S-protein and a monoclonal antibody against vitronectin recognized polypeptides with the same migration on SDS gels. The 65,000- and 75,000-mol wt polypeptides recognized by these antibodies bound avidly to MaVN-Sepharose and heparin-agarose out of serum but not out of plasma. The differential

binding to heparin-agarose is specific to vitronectin/S-protein, as compared with two other heparin-binding glycoproteins, fibronectin or histidine-rich glycoprotein,<sup>20</sup> and is probably due to complex formation with thrombin-anti-thrombin III.<sup>11-13,16</sup> Vitronectin was incorporated into a large complex when serum was activated by zymosan. Finally, we found that MaVN recognized the 65,000- and 75,000-mol wt polypeptides obtained from the purification protocol described by Dahlbäck and Podack for S-protein<sup>14</sup> and that S-protein obtained by this purification was equivalent in spreading factor activity to vitronectin obtained by the purification described by Hayman et al.<sup>4,9</sup>

Jenne and Stanley<sup>17</sup> recently sequenced the cDNA for S-protein obtained from a human liver cDNA pEX library and found it to be similar, although not identical, to the sequence of the cDNA of vitronectin obtained from a human liver cDNA  $\lambda$ gt11 library reported by Suzuki et al.<sup>7</sup> There were discrepancies in the nucleotide sequences of the two proteins that produced differences in amino acids 142-6, 206, 347, 381, and 423.<sup>7,17</sup> There were also conservative nucleo-

Table 1. Spreading Factor Activity of S-Protein

Protein Coating	Cells per mm <sup>2</sup> ( $\bar{x} \pm$ SEM, n = 4)	
	Attached Only	Attached and Spread
S-protein	20 $\pm$ 10	112 $\pm$ 12
Vitronectin	28 $\pm$ 8	117 $\pm$ 12
Fibronectin	47 $\pm$ 14	148 $\pm$ 20
Albumin	67 $\pm$ 13	$\leq 1$

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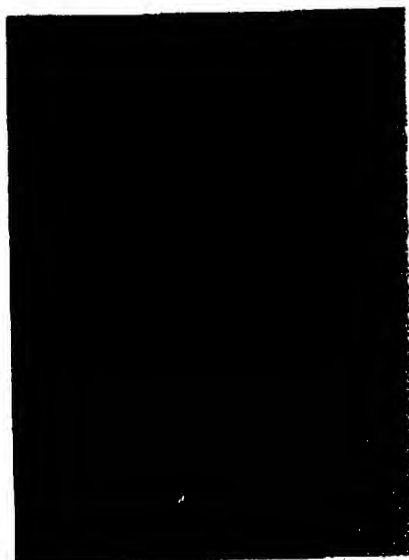


Fig 4. Immunoblot of vitronectin and S-protein digestion by thrombin with MaVN. Vitronectin or S-protein (18  $\mu$ g) were incubated with thrombin (11  $\mu$ g) for two hours at 37 °C. Incubation mixtures were subjected to SDS-PAGE, transferred onto nitrocellulose filters, and treated as described in Materials and Methods with 10% MaVN spent media, followed by 1% goat antimouse peroxidase conjugate. Lane 1, purified vitronectin; lane 2, purified S-protein; lane 3, vitronectin incubated with thrombin; lane 4, S-protein incubated with thrombin. Arrowhead indicates a major proteolytic fragment.

tidic changes. It has been reported that vitronectin is cleaved by thrombin,<sup>22</sup> whereas S-protein is not.<sup>11</sup> However, we found that both proteins are susceptible to thrombin cleavage. Thus, the data summarized in the introduction and presented in Results are strong evidence that vitronectin is the same as S-protein and that the discrepancies in cDNA sequences probably represent sequencing artifacts.

The biological functions of vitronectin/S-protein must be diverse. Others have discussed possible thrombin and trypsin cleavage sites in this protein and the implications for heparin-binding, promotion of cell attachment, and the release of the Somatomedin B polypeptide.<sup>7,17,22</sup> It will be interesting to determine if regulatory conformational changes also enable vitronectin/S-protein to function specifically in different physiological systems. One example of a conformational change that may take place is that apparently induced by binding to the thrombin-antithrombin III complex.<sup>11-13,16</sup> This binding may expose an otherwise buried heparin-binding site in vitronectin.<sup>20,23</sup> Vitronectin/S-protein in the ternary complex could then sequester heparin and thus block the ability of heparin to further accelerate thrombin-antithrombin III complex formation, as reported by Preissner et al.<sup>13</sup>

## ACKNOWLEDGMENT

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# DICTIONARY OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

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- synaptic cleft** See synapse.
- synaptic vesicle** One of a group small vesicles, located in the presynaptic cell of a synapse, that store acetylcholine and play a role in the regulation of acetylcholine within the nerve cell.
- synaptonemal complex** A complex protein structure that forms between, and parallel to, the two paired homologous chromosomes during the early stages of meiosis.
- synaptosome** A largely artificial structure that is produced by disruption of the nerve endings in a synapse. Typically formed by homogenization of brain or spinal cord which results in the snapping off of nerve terminals. The membranes of the latter then reseal to form artifactual, osmotically active organelles that are separable by centrifugation. These structures contain acetylcholine and acetylcholinesterase.
- synarchy** The working together of two inter-related intracellular messengers in regulating various biological functions. The coupling of the actions of cyclic AMP and calcium, that appears to be shared by nearly all differentiated cells of higher organisms, is an example. *Aka* synarchic regulation.
- syncarcinogenesis** Synergistic carcinogenesis.
- syncatalytic process** A process that is synchronous with the catalytic action of an enzyme. A substrate-dependent increase in the reactivity of a functional group of the enzyme or the inactivation of an enzyme by the product of the enzymatic reaction, are two examples.
- synchronous growth** Growth in which all of the cells are at the same stage in cell division at any given time. *Aka* synchronized growth.
- synchronous muscle** A muscle that yields a single contraction for every motor nerve impulse that it receives.
- synchr n us reaction** CONCERTED REACTION.
- synchrotron** An accelerator designed to impart high kinetic energy to charged particles by means of a high-frequency electric field and a low-frequency magnetic field.
- syncytium** A group of cells, joined by cytoplasmic bridges and not separated by cell membranes; an aggregate that contains many nuclei and maintains cytoplasmic continuity.
- syndein** ANKYRIN.
- syndesine** An amino acid that has been isolated from cross-linked collagen chains and that represents the product of an aldol condensation between a molecule of hydroxyallysine and a molecule of allysine.
- syndet** Synthetic detergent.
- syndi tactic p lymer** A polymer in which the R groups of the monomers alternate regularly on both sides of the plane that contains the main chain.
- syndrome** A group of symptoms that occur at the same time and that characterize a disease.
- syneresis** The shrinkage of a gel with the expulsion of liquid. *See also* clot retraction.
- synergism** The phenomenon in which two or more agents work together cooperatively such that their combined effect is greater than the sum of the effects when either agent is acting alone. *See also* substrate synergism.
- synergistic** Of, or pertaining to, synergism.
- synergy** 1. SYNTROPY. 2. SYNERGISM.
- synexin** A protein that occurs in several tissues and causes the  $\text{Ca}^{2+}$ -dependent aggregation of isolated chromaffin granules; believed to promote fusion of the granules with the plasma membrane during exocytosis.
- syngeneic** Referring to genetically identical individuals of the same species; used in reference to tissue transplants.
- syn genes** Mitochondrial genes of yeast that code for mitochondrial protein synthesizing machinery such as tRNA and rRNA.
- syngraft** A transplant from one individual to a genetically identical individual of the same species.
- synhibin** *See* calelectrin.
- synomone** *See* allomone.
- synonym codon** One of several codons that code for the same amino acid, such as the codons UUU and UUC, both of which code for the amino acid phenylalanine.
- synovial fluid** The fluid present at the joints of vertebrates.
- syntenic genes** Genes that are believed to be located on the same chromosome because of their behavior during cell hybridization.
- synthase** 1. LYASE. 2. An enzyme that is not a lyase but for which it is desirable to stress the synthetic aspects of the reaction.
- synthase-phosphorylase kinase** A converter enzyme that catalyzes the interconversion of the two allosteric forms of phosphorylase (a and b); it catalyzes the ATP-dependent phosphorylation of phosphorylase b to a. *Abbr* SPK.
- synthesis** The process whereby a more complex substance is produced from simpler substances by a reaction or a series of reactions; the simpler substances, or portions thereof, are combined to form the more complex substance.
- synthetase** 1. LIGASE. 2. An enzyme that is not a ligase and that catalyzes the formation of a compound by some other mechanism; an example is the enzyme thymidylate synthetase.
- synthetic** 1. Of, or pertaining to, synthesis. 2. Man-made; synthesized in vitro; prepared artificially as opposed to being isolated from natural sources.
- synthetic auxin** A synthetic organic compound